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2014

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citation for published version (APA)

Vosslamber, S. (2014). *The Type I Interferon System In Autoimmune Diseases: Molecular Markers For Heterogeneity And Therapy Response Prediction*. [PhD-Thesis - Research and graduation internal, Vrije Universiteit Amsterdam].

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CHAPTER 3.2

Autoantibody Specificities and Type I IFN Pathway Activation in Idiopathic Inflammatory Myopathies

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To be submitted

ABSTRACT

Background

Idiopathic inflammatory myopathies (IIM) are rare autoimmune disorders characterized by proximal muscle weakness and muscle inflammation. IIM are associated with several autoantibodies, which are believed to play a role in its pathogenesis. Recent studies showed also involvement of an activated type I IFN pathway in a subset of patients. Here we studied the possible relationship between autoantibody specificity and type I IFN pathway activation.

Materials and methods

Ninety-two IIM patients, diagnosed with polymyositis (n=46), dermatomyositis (n=40), or inclusion body myositis (n=6), 47 patients with systemic lupus erythematosus (SLE) and 41 healthy controls (HC) were included. In IIM patients, autoantibody profiles were assessed using lineblots. A whole blood IFN score was determined in all patients and healthy controls by measuring and averaging expression levels of 29 IFN response genes using BioMark™ Dynamic Arrays. Type I IFN bioactivity in serum of 47 IIM patients was determined using a bioassay. The role of IFN α as an interferogenic trigger was determined using neutralizing antibodies in sera of a subset of 25 patients.

Results

The IFN signature was present in 45% of IIM patients, irrespective of their diagnosis. The IFN score was associated with disease activity for patients diagnosed with dermatomyositis but not polymyositis or inclusion body myositis. In IIM patients with a mono-specific autoantibody profile, an association between the presence of an IFN signature and autoantibodies against RNA binding proteins, such as Jo-1, Ro60, SRP and U1RNP, was observed, whereas the absence of the IFN signature is associated with autoantibodies not directed against RNA-binding proteins, such as Ro-52, and PMScl. Moreover, we observed an association between the presence of an IFN signature and multi-specific autoantibody profiles compared to mono-specific autoantibody profiles or absence of autoantibodies (Pearson's Chi square $p = 0.038$ and Pearson Chi square $p=0.002$, respectively). The IFN score correlated with type I IFN pathway-bioactivity (Pearson $r = 0.4243$, $p=0.0057$, $n=47$), which could be partly blocked by neutralizing antibodies directed against IFN α and the type I IFN receptor.

Conclusion

Overall, our findings indicate involvement of IFN α in the type I IFN activity in IIM and suggest a relationship between the presence of anti-RNA-binding protein autoantibodies and the IFN signature in IIM. This hints towards a role for endogenous RNA recognition that triggers type I IFN activity in IIM, similar as has been observed for systemic lupus erythematosus (SLE).

INTRODUCTION

Idiopathic inflammatory myopathies (IIM) are a heterogeneous group of rare autoimmune disorders characterized by proximal muscle weakness and muscle inflammation.¹ An important feature of the disease is the production of autoantibodies, which are present in serum of approximately 80% of the patients diagnosed with polymyositis or dermatomyositis. The autoantibody specificities present in these patients can vary and the role of these different autoantibodies in the pathogenesis of the disease is not fully understood yet. Frequently found antibodies in IIM are anti-Jo-1, anti-Ro52, anti-Ro60 and anti-La, anti-Pm/Scl, anti-SRP and anti-U1RNP. Some autoantibodies are exclusively found in myositis, others are also found in other autoimmune diseases.² The most prevalent antibody in IIM is anti-Jo-1 which specificity is directed against an anti-histidyl tRNA synthetase.³ Anti-Jo-1 is found in 20 - 30% of the IIM patients and associated with anti-synthetase syndrome. Presence of anti-Jo1 can occur years before the onset of clinical symptoms which indicates that they might have a role in the initiation of the disease. Antibodies against the intracellular proteins Ro52, Ro60 and La are also associated with other autoimmune diseases including systemic sclerosis (SS) and systemic lupus erythematosus (SLE) and Sjogren's syndrome. Ro ribonucleoproteins (RNPs) are well-characterized complexes of small cytoplasmic Y RNAs (Y1, Y3, Y4 or Y5 – all RNA polymerase III transcripts).⁴ Most RoRNPs include Ro60, which contains specific Y RNA-binding sites.⁵ The 52 kDa protein Ro-52 is different from Ro-60 in that it lacks such RNA-binding capacities. Monospecificity for anti-Ro-52 (i.e. without anti-Ro-60) is characteristic for a large proportion of patients with IIM and only present at low frequencies in patients with SS and SLE.^{6,7} Anti-PM/Scl antibodies are directed against the PM/Scl complex, also known as the human exosome complex, an RNA processing complex which is located in the nucleolar compartment of the cell.^{8,9} This complex consists of peptides of 75 kDa (PM/Scl-75 protein) and 100 kDa (PM/Scl-100 protein), with PM/Scl-75 being considered the main autoantigen.¹⁰ Of all patients with anti-PM/Scl, 43 to 88% have overlapping syndromes such as scleromyositis or sclerodermatomyositis.^{11,12} The anti-SRP antibody is specific for IIM and directed against the signal recognition particle (SRP).¹³ The signal-recognition particle (SRP) is a cytoplasmic ribonucleoprotein consisting of six polypeptide chains and one molecule of 7SL RNA. SRP is one of the most abundant and best characterized RNP particles and regulates the translocation of proteins across the endoplasmic reticulum during protein synthesis. Anti-U1RNP antibodies are found in many autoimmune diseases, including SLE and IIM. They are directed against U1RNP, which is one of the components of the spliceosome. The U1RNP complex is composed of U1-snRNA, seven common core Sm proteins and three U1-specific proteins (U1-70K, U1-A and U1-C).¹⁴ The presence of specific autoantibodies as well as combinations of autoantibodies has been associated with clinical symptoms of IIM. Another characteristic of IIM is the activated type I interferon (IFN) pathway that has been

observed in a subset of patients. Elevated expression levels of genes related to the type I IFN system including several IFN-response genes (IRGs), as well as IFN β , Toll like receptor (TLR)3, TLR7 and TLR9 were shown in muscle samples of dermatomyositis (DM) and polymyositis (PM) patients.^{15,16,17} Moreover, the MxA protein, a product of one of the type I IFN response genes (IRGs), seems to be present in perifascicular, and sometimes all myofibers, and on capillaries in a majority of DM patients.¹⁵ This local activation of the type I IFN pathway is also reflected in the periphery. Upregulated transcript levels of a large proportion of IRGs, also referred to as the presence of a type I IFN signature, are observed in whole blood cells of a subset of IIM patients in a comparable manner as has been shown for other autoimmune diseases like systemic lupus erythematosus (SLE)¹⁸, rheumatoid arthritis (RA)¹⁹ and multiple sclerosis (MS)²⁰. The signature is present in peripheral blood cells of approximately 50% of the patients studied and the extent of the signature may range from a moderate to high increase. Initial findings in IIM revealed that the presence of a type I IFN signature in the peripheral blood compartment correlated with disease activity.¹⁵ Furthermore, an association with circulating IFN β , but not IFN α or ω , levels was described for IIM.²¹ IFN α levels appear to be reduced in IIM compared to healthy controls.²²

In SLE, the type I IFN signature in the peripheral blood compartment also correlated with disease activity.¹⁸ Pathogenesis of SLE is primarily driven by IFN α . The production of IFN α is a consequence of a continuous activation of plasmacytoid dendritic cells (pDCs) by immune complexes (ICs), consisting of autoantibodies directed against nucleic acids such as anti-ds/ssDNA and anti-RNA, and nucleic acids binding proteins such as anti-sRNP in combination with RNA-containing autoantigens.^{23,24} These interferogenic ICs are internalized via the Fc γ RIIIa expressed on pDCs, reach the endosomes and stimulate TLR7 or 9, which subsequently leads to IFN α gene transcription.²⁵

The similarities between SLE and IIM with respect to the presence of a type I IFN signature and its correlation to disease activity, as well as the presence of autoantibodies in these diseases, suggest similarities in underlying pathology. We speculated that associations between autoantibody specificities and the nature of the IFN signature as observed in SLE may also exist in IIM. Several of the detected autoantibodies in IIM patients are indeed directed against nucleic-acid (RNA)-containing protein complexes. These include e.g. anti-Jo-1, anti-Ro52/Ro60 (but not anti Ro-52 alone), anti-SRP and anti-U1RNP antibodies. However, the relationship between autoantibody profiles and type I IFN signature in IIM has not been examined yet.

In the present study, we investigated the relationship between the presence of autoantibodies directed against RNA binding proteins and other autoantibodies and the type I IFN signature in IIM. Moreover, we studied the nature of the IFN-inducing component that is responsible for the IFN activity in the blood of IIM patients.

We found an association between the whole blood IFN signature in IIM and the presence

of anti-RNA-binding proteins autoantibodies, such as Jo-1, U1RNP and Ro-60. Furthermore, an association between the presence of the IFN signature and multi-specific autoantibody profiles was observed. In analogy to SLE pathogenesis, we provide evidence that IFN α acts as an interferogenic trigger in the serum of IIM patients. Altogether, our results point towards a strong relation between presence of autoantibodies against RNA-binding protein complexes and an IFN α driven type I IFN pathway activation in IIM patients.

PATIENTS AND METHODS

PATIENT RECRUITMENT

IIM patients (n=92) were recruited at the Rheumatology Unit at Karolinska University Hospital in Solna, Stockholm or at the Institute of Rheumatology, Prague. Patients fulfilled the diagnostic criteria for definite or probable PM/DM or sporadic IBM (40 with PM, 46 with DM, and 6 with IBM). Exclusion criteria were presence of overlap syndrome and treatment with biologicals. SLE patients (n=47) were recruited at the Rheumatology department of the VU University medical center. Since SLE patient were used as a reference cohort for IFN score, SLE patients with either high or low disease activity (SLEDAI) were selected. Healthy controls (n=41) were recruited at the VU University medical center. Demographic data, clinical information, and treatment of the patients at the time of serum sampling are shown in Table 1. All patients gave their informed consent.

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Table 1. Patient characteristics

	IIM patients	SLE patients	Healthy Controls
Total amount	92	47	41
Diagnosis, n (%)			
<i>Polymyositis</i>	40 (43)	-	-
<i>Dermatomyositis</i>	46 (50)	-	-
<i>Inclusion Body Myositis</i>	6 (7)	-	-
Age in years, mean (SD)	60(14)	44 (16)	35 ()
Female, %	65	85	56
Disease activity, mean (SD)	n.a.	4 (5)	-
Current Prednisolone use, %	72	50	-
Current use of Immunomodulatory drugs, %	60	63	-

SERUM COLLECTION

Serum was collected and stored at -80°C.

PBMC ISOLATION

Heparinized blood was collected from healthy donors and used for PBMC isolation. PBMCs were isolated by density gradient centrifugation using Lymphoprep (Axis Shield, Oslo, Norway), according to the manufacturer's protocol. After isolation, the PBMCs were cryopreserved in IMDM supplemented with 10% FCS and 10% DMSO and stored in liquid nitrogen until further use.

IRG INDUCTION ASSAY

Healthy PBMCs were incubated with 20% patient serum for 4h or 8h at 37°C and 5% CO₂. Where indicated, samples were co-cultured with 30ng/ml neutralizing anti-IFN α (#21105, PBL, Piscataway, New Jersey, USA) or 2.5 μ g/ml neutralizing anti-IFNAR2 (#21385, PBL, Piscataway, New Jersey, USA). After incubation, cells were harvested, washed and lysed in RLT buffer (Qiagen Benelux BV, Venlo, The Netherlands) according to the manufacturer's protocol. The lysates were stored at -20°C until RNA isolation.

RNA ISOLATION FROM CULTURES CELLS

RNA was isolated from the cell lysates using the RNeasy Micro kit (Qiagen Benelux BV, Venlo, The Netherlands), according to the manufacturer's protocol. A DNase (Qiagen Benelux BV, Venlo, The Netherlands) step was included to remove genomic DNA. Quantity and purity of the RNA was determined using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, Delaware USA). 50ng of RNA was used for cDNA synthesis, which was performed using the a Revertaid H-minus cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany), according to the manufacturer's protocol.

WHOLE BLOOD SAMPLING AND RNA ISOLATION

For whole blood RNA isolation, 2.5 ml blood was drawn in PAXgene tubes (PreAnalytix, GmbH, Germany), incubated 2 hours at room temperature and stored at -20°C. Tubes were thawed overnight at room temperature prior to RNA isolation. Total RNA was isolated according to the manufacturer's instructions (PAXgene Blood RNA Mdx kit). Total RNA concentration was measured using the Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE) and RNA quality was assessed based on 260nm/280nm.

REVERSE TRANSCRIPTION OF CDNA

RNA (0.5 μ g whole blood RNA derived from PAXgene tubes or 50ng RNA derived from cultures cells) was reverse transcribed into cDNA using a Revertaid H-minus cDNA synthesis

kit (MBI Fermentas, St. Leon-Rot, Germany) according to the manufacturers' instructions.

REAL TIME QPCR

IFN response gene (IRG) expression was determined in mRNA derived from cultures cells in the bioassay using Real Time Quantitative PCR (qPCR). qPCR was performed using SYBRGreen (Applied Biosystems, Foster City, CA, USA) and an ABI Prism7500HT Sequence detection system (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's protocols. Primers were designed using Primer Express software and guidelines (Applied Biosystems, Foster City, CA, USA). To calculate arbitrary values of mRNA levels and to correct for differences in primer efficiencies a standard curve was constructed. Expression levels of target genes were calculated relative to housekeeping gene 18S ribosomal RNA (18SrRNA). To correct for any variation between experiments, all expression values are shown relative to untreated NHS. The overall IRG induction was determined by calculating the average expression of three known IRGs; RSAD2, IFI44L and MX1.

MULTIPLEX REAL-TIME PCR ARRAYS

On the PAXgene derived whole blood RNA, Real-Time PCR analysis was performed at ServiceXS (ServiceXS B.V., Leiden, The Netherlands) using the 96.96 BioMark™ Dynamic Array for Real-Time PCR (Fluidigm Corporation, San Francisco, CA, U.S.A), according to the manufacturer's instructions. Before use on the BioMark array, the cDNA was 1:1 diluted in nuclease-free water and subjected to 14 cycles of Specific Target Amplification using a 0.2X mixture of all Taqman Gene Expression assays in combination with the Taqman PreAmp Master Mix (Applied Biosystems), followed by 5-fold dilution. Thermal cycling and real-time imaging of the BioMark array was done on the BioMark instrument, and Ct values were extracted using the BioMark Real-Time PCR analysis software. Data was processed by automatic global threshold setting with the same threshold value for all assays and linear baseline correction using BioMark Real-time PCR Analysis software (version 2.1.1). The quality threshold was set at the default setting of 0.65.

Relative quantities were calculated using the ddCT method. GAPDH was used as a housekeeping gene and all arrays contain 2 samples for calibration.

CALCULATION OF INTERFERON (IFN) SCORE

In HCs (n=41), SLE (n=47) and IIM (n=94), the average expression of twenty-nine known and highly correlating IFN response genes (table 2) (all corrected versus GAPDH, log2) was calculated and referred to as IFN score. The mean + 2*SD of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84).

AUTOANTIBODY PROFILING

Serological data was obtained using line-immunoassay system (Euroimmun, Lubeck, Germany). This assay detects autoantibodies directed to Jo-1, Mi2, Ku, PM-Scl, PM-Scl75, PM-Scl100, PL-7, PL-12, Ej, Oj, SRP, Ro, Ro52, La, Scl70 and U1-snRNP antigens.

STATISTICAL ANALYSIS

IFN scores in groups of patients were checked for Gaussian distribution according to Kolmogorov-Smirnov test and for comparison of IFN scores between groups of patients students t test or Mann Whitney t test were performed, if appropriate. Differences in IRG induction over time were tested using a paired t test. For comparison of the number of IFN signature positive patients per group, Pearson's Chi square tests were performed. Correlation analyses were performed using Pearson r or Spearman r tests. All analyses were performed using Graphpad Prism 4 software.

RESULTS

WHOLE BLOOD IFN SIGNATURE IN HC, SLE AND IIM SUBTYPES

To examine the extent of the IFN signature in whole blood RNA samples of IIM patients, SLE patients and healthy controls (HC) an IFN-score was calculated. The IFN score as well as the presence or absence of an IFN signature was used in the analyses, if applicable. The

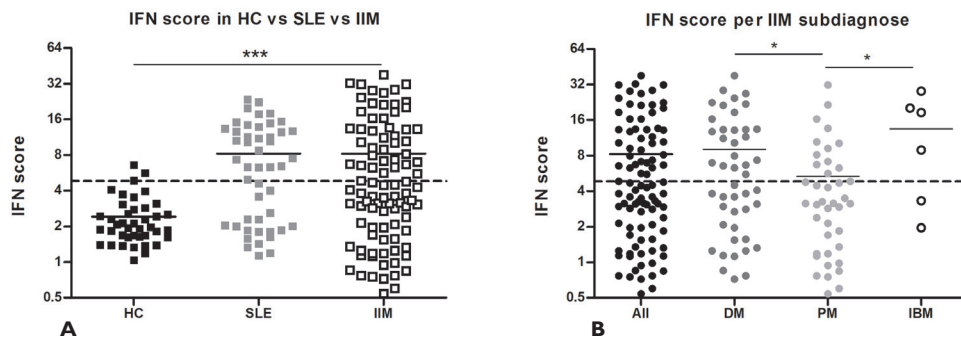


Figure 1. The IFN score in IIM patients, SLE patients and healthy controls.

Gene expression levels of 29 interferon-response genes were averaged to calculate the IFN score. The IFN score was measured whole blood cells of IIM patients. The mean + 2*SD of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line).. **A)** The extent of the IFN score was compared between HCs, SLE and IIM patients and an increased IFN score was observed in a subset of SLE and IIM patients. **B)** The extent of the IFN score was compared between IIM patients with different subdiagnosis, e.g. dermatomyositis (DM), polymyositis (PM) and Inclusionbody myositis (IBM). An IFN signature was observed in a subset of patients, irrespective of their subdiagnosis, but IFN score was higher in DM and IBM patients compared to PM patients (Mann Whitney p= 0.0415 and 0.0415 respectively).

mean + 2*SD of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84). As expected, the observed IFN score is significantly higher in IIM patients (average IFN score = 8.23) compared to healthy controls (average IFN score 2.41) (Mann Whitney t test, $p = 0.0007$) and reached expression levels comparable to those in SLE patients (average IFN score 8.22) (Figure 1a). The IFN signature was present in whole blood of a subgroup of IIM patients (45%), irrespective of diagnosis. Moreover, a correlation was observed between the extent of the IFN score and disease activity (MYOAct global) for patients diagnosed with dermatomyositis (Spearman $p = 0.03$, $n = 32$) but not polymyositis and inclusion body myositis (Figure 1B).

THE IFN SIGNATURE IN RELATION TO AUTOANTIBODIES IN IIM

In order to search for a relation between the differential activation of type I IFN pathway and presence of autoantibodies in IIM, the association between the IFN signature and autoantibody profiles was tested. No association between autoantibody positivity and the presence or extent of the IFN signature was revealed (Figure 2A). Next we studied whether the IFN signature was associated with autoantibody specific status in IIM. Twenty IIM patients have a multi-specific autoantibody status, of which 14 contained an IFN signature (70%), which was significantly more frequent than in patients with only one autoantibody

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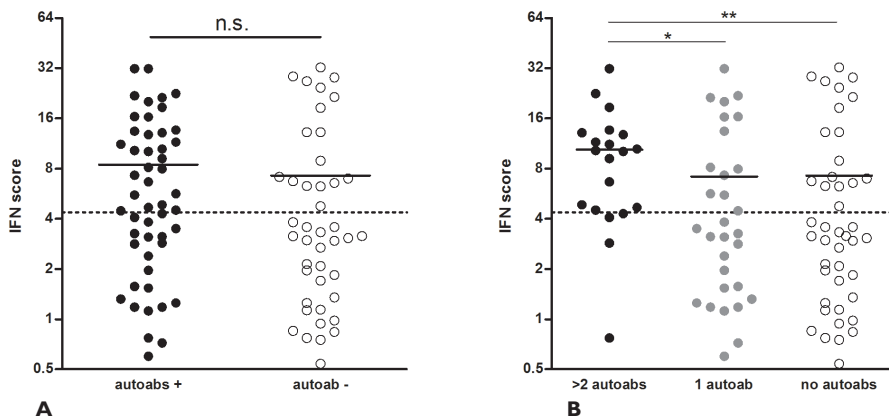


Figure 2. IFN score in IIM patients with and without autoantibodies.

Gene expression levels of 29 interferon-response genes were averaged to calculate the IFN score. The IFN score was measured whole blood cells of IIM patients. The mean + 2*SD of the IFN score in healthy controls (n=41) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line). **A)** IFN score was compared between IIM patients with and without autoantibodies. No differences in extent or presence of IFN signature were observed. **B)** IFN score was compared between patients with multi-specific autoantibody profiles versus patients with mono-specific autoantibody profiles and patients without autoantibodies. Almost all patients with multispecific autoantibody profiles have an IFN signature and the IFN score was higher in those patients compared to patients with mono-specific autoantibody profiles and patients without autoantibodies (Mann Whitney $p = 0.0240$ and $p = 0.0098$ respectively).

specificity (18 out of 30 (60%)) (Pearson's Chi square $p = 0.038$) and patients without specific autoantibodies (27 out of 43 (63%)), (Pearson Chi square $p=0.002$). In addition, a higher IFN score was observed in the patients with multi-specific autoantibody status compared to those with only one (Mann Whitney $p=0.024$) or no (Mann Whitney $p=0.0098$) autoantibody specificity (Figure 2B). These findings indicate that the presence of multiple autoantibody specificities is related and may contribute to the IFN signature.

IFN SIGNATURE IS RELATED TO AUTOANTIBODIES AGAINST RNA-BINDING PROTEIN COMPLEXES IN IIM PATIENTS

In order to search for a relation between the differential activation of type I IFN pathway and presence of autoantibody specificities in IIM, we initially analyzed the association between the IFN signature and the presence of anti-nuclear antibodies (ANA) as a whole. No significant difference in the IFN score nor the presence of the IFN signature was observed between ANA negative ($n=44$) and ANA positive ($n=50$) patients (data not shown).

To determine whether the IFN signature is associated with more distinct autoantibody

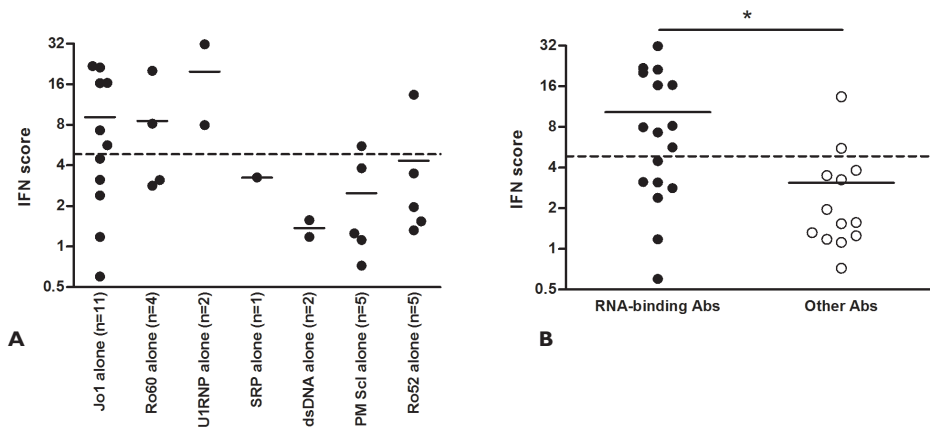


Figure 3. IFN score in IIM patients with mono-specific autoantibodies directed against RNA-binding proteins or non-RNA binding proteins.

Gene expression levels of 29 interferon-response genes were averaged to calculate the IFN score. The IFN score was measured in whole blood cells of IIM patients. The mean + 2*SD of the IFN score in healthy controls ($n=41$) was used as a cut off to define if an IFN signature was present (IFN score > 4.84) or absent (IFN score < 4.84) (dotted line). The association between the IFN score and monospecificity of autoantibodies is depicted and the results suggest an association between the presence of an IFN signature and RNA binding proteins such as anti-Jo-1, anti-Ro60, and anti-U1RNP. **A)** Patients were grouped according their mono-specific autoantibody profile. Presence of an IFN signature (IFN score above 4.84) was mainly observed in patients positive for antibodies against RNA-protein complexes anti-Jo-1 (55%), anti-Ro60 (50%) and anti-U1RNP (100%) and to a much lesser extend in patients positive for autoantibodies not directed against such complexes, e.g. anti-dsDNA 90%, anti-PM/Scl (20%) and anti-Ro52 (20%). **B)** IFN score and signature were associated with autoantibodies directed against RNA-binding protein complexes (Mann Whitney t test $p=0.011$, Pearsons Chi square, $p=0.003$).

specificities in IIM, patients with a mono-specific autoantibody status were selected (n=30). The IFN signature was clearly present in patients with mono-specificity for anti-Jo-1 (55% positive for IFN signature), anti-Ro60 (50%) and anti-U1RNP (100%) autoantibodies and absent in most patients with mono-specificity for anti-Ro52 (20%), anti-PM/Scl (20%) or anti-dsDNA (0%) antibodies (Figure 3A). Since anti-Jo1, anti-Ro60 and anti-U1RNP antibodies are directed against RNA-containing protein complexes, patients were separated based on their autoantibody specificity, i.e. directed against RNA-containing protein complexes (anti-Jo-1, anti-Ro60, anti-U1RNP, anti-SRP) versus other autoantibodies (anti-dsDNA, anti-PM Scl, anti-Ro52). This analysis revealed that the IFN signature is associated with autoantibodies directed against RNA-binding protein complexes (Mann Whitney t test $p=0.011$, Pearson's Chi square, $p=0.003$) (Figure 3B).

IFN α IN IIM PATIENTS' SERUM IS RESPONSIBLE FOR TYPE I IFN PATHWAY ACTIVATION

To search for a possible trigger or interferogenic component in the IIM patients' serum, we analyzed the capacity of IIM patient serum to activate the type I IFN pathway. Upregulation of IRG expression was determined as an outcome measure for type I IFN pathway-activating capacity. A subset of the IIM patients' serum (n=10) showed direct type I IFN pathway-activating capacity four hours after serum-addition (Figure 4A, 'IRG high') whereas the serum of other patients showed no IRG induction (Figure 4A, 'IRG low') (Student t test, $p<0.0001$, (average IRG induction (1.82) was used to subdivide IFN^{high} and IFN^{low} patients (Figure 4A)). None of the patients' sera induced IRG expression at eight hours after serum-addition (Figure

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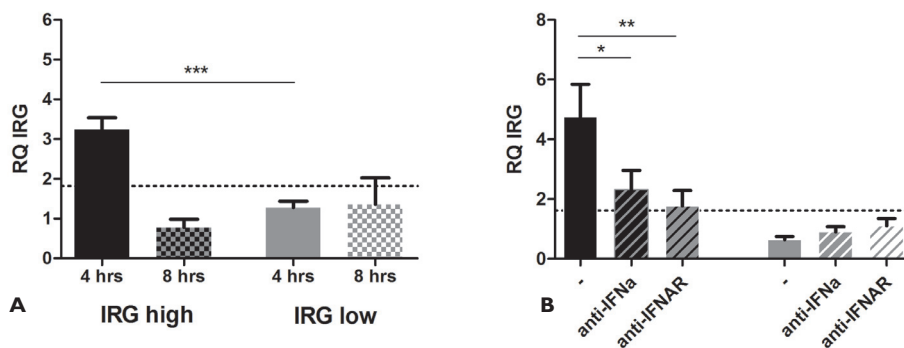


Figure 4. IFN bioactivity in serum of IIM patients.

A) Serum-induced IRG expression after 4 and 8 hours. Sera of IRG high IIM patients induces IRG expression in healthy PBMCs at four hours after incubation. This IRG induction was not observed in other patients' sera (IRG low) (Student t test, $p<0.0001$, (average IRG induction (1.82) was used to subdivide IFN^{high} and IFN^{low} patients). After eight hours incubation, in none of the patients sera IRG expression was observed. **B) Blocking of serum-induced IRG expression.** Serum-induced IRG expression was blocked when anti-IFN α or anti-IFNAR were added to the serum. A significant effect was observed for both anti-IFN α and anti-IFNAR antibodies IRG high patients (Paired t test $p=0.0160$ and $p=0.0095$, respectively), indicating that IFN α was responsible for the observed IRG induction.

4A). Serum-induced IRG expression after four hours of incubation positively correlates with the IFN signature in whole blood (Pearson $r=0.4$, $p=0.005$). Similar observations were made with serum from SLE patients (data not shown). This suggests the presence of a direct trigger for IFN pathway activation in serum that is responsible for IRG induction.

To investigate whether type I IFNs are responsible for the observed activation of the type I IFN pathway in a subset of IIM patients ($n=25$), anti-IFNAR antibodies were added to the sera and the type I IFN bioactivity was determined after 4 hours. Indeed in IRG high patients, IFN bioactivity was inhibited by anti-IFNAR (Paired t test $p=0.0160$, Figure 4b). Subsequently, we tested whether IFN α could be held responsible for the induction of the type I bioactivity in IIM serum, in analogy to SLE. This analysis revealed that addition of anti-IFN α antibodies inhibited the capacity to induce type I IFN bioactivity in IIM serum (Paired t test $p=0.0095$, Figure 4B). These data suggest the presence of IFN α in the serum of IIM patients as an endogenous factor that can trigger the type I IFN pathway resulting in an IFN signature in these patients.

DISCUSSION

The results in the present study demonstrate an association between the activation of the type I IFN pathway in IIM patients and the presence of autoantibodies against RNA containing protein complexes, such as anti-Jo-1, anti-U1RNP and anti-Ro-60. This association is stronger in patients with more than one autoantibody specificity. Evidence is generated that IFN α is present in the serum of IIM patients and is likely to be involved as activator of the type I IFN pathway. These findings indicate (partly) comparable mechanisms underlying the regulation of IFN type I activity in SLE and IIM.

Accumulating evidence suggests a role for type I IFNs in the pathogenesis of IIM. In fact, already in the 1980's, the presence of type I IFN proteins was observed in muscle biopsies of patients.²⁶ In addition, several cases of patients developing autoimmune symptoms including DM and PM characteristics after treatment with IFN α or IFN β have been reported.^{27,28,29,30} More recently, IRGs were shown to be upregulated in muscle (and skin) biopsies of DM patients and the type I IFN signature was shown to be present in blood samples of a majority of these patients.^{15,16,17} A correlation between IFN signature and disease activity in patients diagnosed with DM further supports the hypothesis of a role of the type I IFN system in IIM.¹⁶ The exact relationship between local IFN and related symptoms, and the IFN signature in the periphery remains unknown as well as the mechanism behind activation of the type I IFN pathway in IIM. The presence of type I IFNs in IIM patients sera was also described by Liao et al.²¹, who reported a correlation between IFN β protein, and not IFN α or IFN ω in serum or plasma and IRG expression levels of paired PBMCs. Moreover, results of Wong

et al.³¹ indicated a strong relation between IFN β and the IFN signature in muscle and skin biopsies of IIM patients.

As mentioned before, several similarities were observed with respect to type I IFN activation in SLE and IIM. In the present study, we observed a correlation between whole blood IFN signature and disease activity in IIM, as described previously by Welsch et al. This is an indication that there might be a causal relation between type I IFN activation and disease manifestation in IIM, as was observed in SLE before. Subsequently, we showed a correlation between the IFN signature in the whole blood and *in vitro* type I IFN inducing capacity of the serum in IIM. The results showed an induction at 4 hrs but not at 8 hrs, suggesting that the IRG induction is a rapid process and that the induction is most likely due to a direct effect of a serum component that binds to the IFNAR and activates the JAK/STAT pathway. We were able to demonstrate a role for an IFNAR driven response by showing that addition of blocking anti-IFNAR antibodies inhibited the IFN bioactivity in serum of IIM patients. Subsequently, we were able to show that IFN α present in the serum of IIM patients was responsible for the observed *in vitro* IRG induction. Thus it may be supposed that besides IFN β , also IFN α protein is present in the serum of IIM patients, which is important for the type I IFN activation in IIM and in line with observations reported for SLE.³²

Another important parallel between SLE and IIM is the association between type I IFN activity and the presence of antibodies directed against RNA-binding complexes. We demonstrated that the *in vivo* IFN signature is mainly present in patient with anti-Jo-1, anti-U1RNP or anti-Ro60 and to a much lesser extent in patients with other autoantibodies tested. The common denominator of these antibodies that associate with the IFN signature is that they are directed against RNA-binding protein complexes. Anti-Jo-1 is an antibody specific for IIM and is the most prevalent antibody in IIM. It is directed against an anti-histidyl RNA synthetase¹³, an enzyme that catalyzes the formation of aminoacyl-tRNA after which the amino acid is transferred by the ribosome onto a growing peptide. The complex of enzyme and tRNA that is bound by the anti-Jo-1 antibodies thus involves RNA. Anti-U1RNP, an antibody that is also characteristic for other autoimmune diseases, is directed against a component of the spliceosome, a complex which also contains snRNA. This antibody is also associated with an activated type I IFN pathway in SLE patients.³³ The antibodies against Ro60 and Ro52 are also associated with (among others) SLE. Ro ribonucleoproteins (RNPs) are complexes of small cytoplasmic Y RNAs and most RoRNPs include Ro60, which contains specific Y RNA-binding sites.⁵ Ro 52 can bind to this RNA-containing complex via Ro60 and those antibodies are often both present in a patient. However, Ro-52 is different from Ro-60 in that it lacks RNA-binding capacities and does on itself not bind to an RNA-containing complex. Monospecificity for anti-Ro-52 (i.e. without anti-Ro-60) is characteristic for a large proportion of patients with IIM, but only present at low frequencies in patients with SS and SLE.^{34,35} Our results indeed show that only a minority of patients monospecific for Ro52 are

positive for an IFN signature. However, Ro52 is involved in the regulation of the type I IFN pathway since Ro52 is both an IFN-inducible protein as well as a regulator of IFN responses via its promoting and inhibitory effects on IRFs.^{36,37,38} Whether or not this effect on the IFN signalling is relevant in these patients, needs to be further elucidated. Antibodies against SRP are also directed against an RNA-binding complex. SRP is a cytoplasmic ribonucleoprotein and contains 7SL RNA. It is one of the most abundant and best characterized RNP particles. However, our study did not include enough patients with monospecific positivity for anti-SRP to draw any conclusions about the association of this specific antibody and the IFN signature.

Our data is compatible with results from Eloranta and colleagues who showed the *in vitro* IFN-inducing capacity of IIM patients' sera containing anti-Jo-1 and/or anti-Ro52/Ro60.³⁹ Eloranta et al. used a bioassay in which they added serum to healthy control PBMCs in the presence of necrotic material to study the capacity of anti-Jo-1 and anti-Ro52/Ro60 antigen containing ICs to induce IFN α protein production. They showed the capacity to induce IFN α only in cultures where both anti-Jo-1 or anti-Ro52/Ro60 and necrotic material was present. Our observation that presence of the IFN signature is associated with IFN α driven bioactivity and anti-Jo-1 and anti-Ro52/Ro60 positivity demonstrates the physiological relevance of these observations.

Altogether, our results imply that, in line with observations in SLE, anti-RNA binding antibodies are associated with activation of the type I IFN pathway in IIM via upregulation of at least IFN α protein. Since in SLE immune complexes, consisting of autoantibodies, autoantigens and RNA can bind to and are internalized by Fc γ receptors on pDCs, leading to activation of endogenous TLR7 or TLR9 and subsequent production of IFN α , it is tempting to speculate that a similar pathogenesis as is observed in SLE is at least partly active in IIM, which may have implications for the treatment and subclassification of these disorders.

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